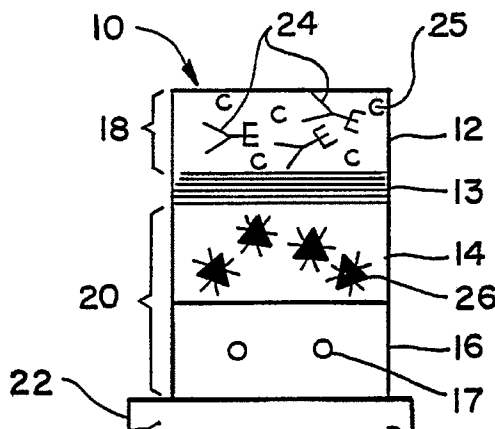




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: LAYERED IMMUNOASSAY DEVICE CONTAINING FLUID FLOW BARRIER



## (57) Abstract

The present invention is directed to improved method and device for detecting the presence of antigens in sample fluids which comprises bringing fluid to be tested for presence of antigens into contact with the device having first reactive zone (18), enzyme regulated fluid flow barrier (13), and second reactive zone (20). The first reactive zone (18) is composed of matrix layer (12) containing bacteria collagenase and enzyme-linked antibodies (24) which are capable of immunologically reacting with antigens being tested for. An enzyme regulated fluid flow barrier (13) is operably connected to first reactive zone (18) of the device and the barrier (13) is composed of sufficient amount of collagen to allow fluid flow when degraded by bacterial collagenase of first reactive zone. A second reactive zone (20), having two interconnecting matrix layers (14, 16), i.e. trapping layer (14) and substrate layer (16), is also operably connected to fluid flow barrier (13) of the device. The trapping layer (14) contains bound and immobilized antigens which are recognized by enzyme-linked antibodies (24) of first reactive zone (18), and substrate layer (16) contains material capable of reacting with enzyme linked to enzyme-linked antibodies (24) of first reactive zone (18) to produce a color forming reaction. After sufficient period of time for allowing the sample fluid to permeate the device, the presence or absence of any color change in the second reactive zone is observed to determine the presence of the antigens being tested for the sample fluid.

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LAYERED IMMUNOASSAY DEVICE CONTAINING  
FLUID FLOW BARRIER

Background of the Invention

The present invention relates to an improved device and method for determining the presence of antigens and/or antibodies in sample fluids. More particularly, the present invention is directed to improvements in the immunoassay field, specifically in layered immunoassays containing immobilized antigens such as that described in Liotta, U.S. Patent No. 4,446,232.

The Liotta '232 patent discloses an immunoassay device fabricated out of matrix material having a first layer or zone impregnated with specific enzyme-linked antibodies which are capable of immunologically reacting with the antigens being tested for, a trapping layer or zone containing bound and immobilized antigens which are of the type recognized by the antibodies of the first layer, and a third layer or zone containing a color forming substrate which reacts with the enzyme linked to the antibodies. When a sample fluid containing the antigens to be tested for is placed in contact with the first layer, the free antigens in the sample combine with the enzyme-linked antibodies of the first layer to form antigen-antibody(enzyme-linked) complexes which freely diffuse through the trapping layer into the third layer to produce a color reaction. If the fluid sample contains no antigens, all of the enzyme-linked antibodies will have free binding sites and will combine with the immobilized antigens present in the trapping layer, and no color reaction will be produced.

However, while satisfactory for most purposes, it has been discovered that in certain circumstances (such as for antigens in very low concentrations) the device as originally described by the Liotta patent does not have the

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desired degree of sensitivity. This is because the reaction between the free antigens in the test sample and enzyme-linked antibodies present in the first layer occurs as the fluid passively flows or permeates through the test strip. As a result of this fairly quick fluid flow process, antigens in low abundance may not have enough time to react sufficiently with the enzyme linked antibodies.

In order to improve the sensitivity of the original device, Dr. Liotta, the present, as well as the original inventor of the Liotta immunoassay, has done a great deal of research in order to develop a built-in delay mechanism which would allow the free antigens present in the sample fluid and the enzyme-linked antibodies of the first layer to react for a longer period of time before entering the trapping layer. It has been discovered that as a result of this increased incubation period, a greater number of antigen-antibody(enzyme-linked) complexes can be formed, thereby increasing the color forming reaction produced in the substrate zone and in turn improving the sensitivity of the assay.

The present invention concerns the addition of an enzymatically regulated fluid flow barrier between the first reactive zone and the trapping layer of the immunoassay to act as such a delay mechanism. The enzymatically regulated fluid flow barrier increases the incubation period for the formation of the antigen-antibody(enzyme-linked) complexes in the first reactive zone, and overcomes the problem of short unregulated incubation time in the first zone of the Liotta device as it was originally described, resulting in an immunoassay exhibiting improved sensitivity.

The foregoing and other advantages of the instant

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invention will become apparent to those skilled in the art from a reading of the following specification and claims.

#### Summary of the Invention

In one aspect, the present invention concerns a device for detecting the presence of antigens in a sample fluid which comprises a first reactive zone containing bacterial collagenase and enzyme-labeled antibodies which are capable of immunologically reacting with the antigens being tested for. A protease regulated fluid flow barrier is operably connected to the first reactive zone of the device and the barrier is composed of a sufficient amount of collagen to allow fluid flow only when degraded by the bacterial collagenase of the first reactive zone. A second reactive zone, having two interconnecting matrix layers, i.e. a trapping layer and a substrate layer is also operably connected to the fluid flow barrier of the device. The trapping layer contains bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone. The substrate layer contains material capable of reacting with the enzyme linked to the enzyme-linked antibodies of the first reactive zone to produce a color forming reaction.

In another aspect, the present invention, is directed to a unique method for detecting the presence of antigens in a sample fluid which comprises bringing a fluid which is to be tested for the presence of antigens into contact with a device having a first reactive zone, an enzyme regulated fluid flow barrier, and a second reactive zone. The first reactive zone is composed of a matrix layer or reservoir containing dried or lyophilized bacterial collagenase and enzyme-linked or otherwise labeled antibodies which are capable of immunologically

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reacting with the antigens being tested for. A protease regulated fluid flow barrier is operably connected to the first reactive zone of the device and the barrier is composed of a sufficient amount of collagen to allow fluid flow only when sufficiently degraded by the bacterial collagenase of the first reactive zone. A second reactive zone, having two interconnecting matrix layers, i.e. a trapping layer and a substrate layer, is also operably connected to the fluid flow barrier of the device. The trapping layer contains bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone and the substrate layer contains material capable of reacting with the enzyme linked to the antibodies of the first reactive zone to produce a color forming reaction. After a sufficient period of time for allowing the sample to permeate the device, the presence or absence of any color change in the second reactive zone determine the presence or absence of the antigens being tested for in the sample fluid.

In a still another aspect, the present invention concerns a device for detecting the presence of antibodies in a sample fluid comprising a first reactive zone containing bacterial collagenase and enzyme-linked antigens which are capable of immunologically reacting with the antibodies being tested for. A protease regulated fluid flow barrier is operably connected to the first reactive zone of the device, and the barrier is composed of a sufficient amount of collagen to allow fluid flow only when degraded by the bacterial collagenase of the first reactive zone. A second reactive zone having two interconnecting matrix layers, i.e. a trapping layer and a substrate layer, is also operably connected to the fluid flow barrier of the device. The trapping layer contains bound and immobilized antibodies which are of the type recognized by the enzyme-

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linked antigens of the first reactive zone. The substrate layer contains material capable of reacting with the enzyme linked to the antigens of the first reactive zone to produce a color forming reaction.

5 In a still further aspect, the instant invention is directed to a unique method for detecting the presence of antibodies in a sample fluid which comprises bringing a fluid which is to be tested for the presence of antibodies into contact with a device having a first reactive zone, an  
10 enzyme regulated fluid flow barrier, and a second reactive zone. The first reactive zone is composed of a matrix layer or reservoir containing bacterial collagenase and enzyme-linked antigens which are capable of immunologically reacting with the antibodies being tested for. A protease  
15 regulated fluid flow barrier is operably connected to the first reactive zone of the device. The barrier is composed of a sufficient amount of collagen to allow fluid flow only when degraded by the bacterial collagenase of the first reactive zone. A second reactive zone, having two  
20 interconnecting layers, i.e. a trapping layer and a substrate layer, is also operably connected to the fluid flow barrier. The trapping layer contains bound and immobilized antibodies which are of the type recognized by the enzyme-linked antigens of the first reactive zone and  
25 the substrate layer contains material capable of reacting with the enzyme linked to the enzyme-linked antigens of the first reactive zone to produce a color forming reaction. After a sufficient period of time for allowing the sample fluid to permeate the device, the presence of absence of  
30 any color change in the second reactive zone is observed to determine the presence of absence of the antibodies being tested for in the sample fluid.

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Description of the Drawings

The following is a brief description of the drawings which are presented for the purpose of illustrating the invention and not for the purpose of limiting same.

5           FIGURE 1 is a diagrammatical illustration of the device 10 which may be utilized to detect the presence of antigens in sample fluids. Device 10 is constructed in four distinct layers, 12, 13, 14, and 16. Layer 12 forms the first reactive zone 18 and is fashioned from a porous material which has dispersed therein, enzyme-linked  
10           antibodies 24, which are capable of immunologically reacting with the antigen being tested for, and bacterial collagenase 25. Layer 13 is an enzyme regulated fluid flow barrier which separates the first reactive zone 18 from the  
15           second reactive zone 20 and is composed, at least in part, of collagen. Layers 14 and 16 form the second reactive zone 20. Layer 14 is formed of a porous material and has bound thereto antigens 26 which are of the type recognized by enzyme-linked antibodies 24. Layer 16 is also  
20           fabricated from a porous material and contains a color forming reagent 17 which generates a signal in the presence of the enzyme linked to the antibodies 24. The device 10 is shown positioned on a supporting member 22.

25           FIGURES 2A, 2B, and 2C are diagrammatical illustrations showing the utilization of the device of FIGURE 1 for the detection of antigens in sample fluids. Specifically, in FIGURE 2A, a sample fluid, generally identified by the numeral 28, containing free antigens 32, is applied to the surface 30 of layer 12. As the sample  
30           fluid permeates through the matrix of layer 12, the enzyme-linked antibodies 24 and the bacterial collagenase 25 become solubilized. Free antigens 32, of the sample fluid



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28, then contact and bind to some of the soluble enzyme-linked antibodies 24 present in layer 12 to form antigen-antibody(enzyme-linked) complexes 34 with saturated recognition sites. The collagen fluid flow barrier 13 prevents the fluid containing the free antigens 32, the soluble enzyme-linked antibodies 24 and the antigen-antibody(enzyme-linked) complexes 34 from diffusing into layer 14. As a result of the increased incubation period due to the collagen fluid flow barrier 13, additional antigen-antibody(enzyme-linked) complexes 34 with saturated recognition sites are formed in layer 12 while the bacterial collagenase 25 begins to degrade the collagen fluid flow barrier 13.

After a short degradation period, the collagen fluid flow barrier 13 is digested by the bacterial collagenase 25, allowing the fluid containing the soluble enzyme-linked antibodies 24 and the antigen-antibody(enzyme-linked) complexes 34 to diffuse into the second reactive zone 20 (or layer 14) (FIGURE 2B), where the remaining enzyme-linked antibodies 24 not complexed with antigens 32 of the sample fluid become attached to the immobilized antigens 26. The fluid containing the antigen-antibody(enzyme-linked) complexes 34 having saturated recognition sites then freely diffuses through layer 14 and enters layer 16 where the enzyme linked to the antibodies of the antigen-antibody complexes 34 reacts with the color forming reagent 17 to produce a color which indicates the presence of the antigens 32 in the sample (FIGURE 2C).

FIGURES 3A, 3B, and 3C are a diagrammatical illustration showing the method of the invention using the device of FIGURE 1 and showing what happens when the test fluid is devoid of antigens. Specifically, a fluid, generally designated 34, is applied to the surface 30 of layer 12. As the fluid diffuses through the matrix of

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layer 12, the enzyme-linked antibodies 24 and the bacterial collagenase 25 are solubilized (FIGURE 3A). After a short degradation period, the collagen fluid flow barrier 13 is digested by the bacterial collagenase 25, allowing the fluid containing the soluble enzyme-linked antibodies 24 to diffuse into layer 14 (FIGURE 3B), where the enzyme-linked antibodies 24 become attached to the bound and immobilized antigens 26. Accordingly, no enzyme-linked antibodies 24 reach the color forming reagent 17 in the second zone 20 (layer 16) and no color change is observed (FIGURE 3C), indicating that the sample fluid 34 is devoid of antigen.

FIGURE 4 is a diagrammatic illustration of device 40, which is an alternative embodiment of the present invention. Device 40 may be utilized to detect the presence of antibodies as opposed to antigens in sample fluids and is constructed of the same layers and zones as in device 10. However, device 40 contains in layer 12, enzyme-linked antigens 36, and not the enzyme-linked antibodies of device 10. In addition, device 40 contains in layer 14, bound antibodies 38, and not the bound antigens of device 10.

FIGURES 5A, 5B, and 5C are diagrammatical illustrations showing the utilization of the device of FIGURE 4 for the detection of antibodies in sample fluids. Specifically, in FIGURE 5A, a sample fluid, generally identified by the numeral 42, containing free antibodies 44, is applied to the surface of layer 12. As the sample fluid diffuses through the matrix of layer 12, the enzyme-linked antigens 36 and the bacterial collagenase 25 become solubilized. Free antibodies 44, of the sample fluid 42, then contact and bind with most the soluble enzyme-linked antigens 36 present in layer 12 to form antibody-antigen(enzyme-linked) complexes 46 with saturated recognition sites. The collagen fluid flow barrier 13

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prevents the fluid containing the free antibodies 44, the soluble enzyme-linked antigens 36 and the antibody-antigen(enzyme-linked) complexes 46 from diffusing into layer 14. As a result of the increased incubation period due to the collagen fluid flow barrier 13, additional antibody-antigen(enzyme-linked) complexes 46 with saturated recognition sites are formed in layer 12 while the bacterial collagenase 25 begins to degrade the collagen fluid flow barrier.

After a relatively short degradation period, the collagen fluid flow barrier 13 is digested by the bacterial collagenase 25, allowing the fluid containing the soluble enzyme-linked antigens 36 and the antibody-antigen(enzyme-linked) complexes 46 with saturated recognition sites to diffuse into layer 14 (FIGURE 5B), where the remaining enzyme-linked antigens 36 become attached to the bound antibodies 38. The fluid containing the antibody-antigen(enzyme-linked) complexes 46 having saturated regulated sites then freely diffuses through layer 14 and enters layer 16 where the enzyme-linked to the antigen of the antibody-antigen complexes 46 reacts with the color forming reagent 17 to produce a color which indicates the presence of the antibodies 44 in the sample (FIGURE 5C).

FIGURES 6A, 6B, and 6C are diagrammatical illustrations of the method of the invention using the device of FIGURE 4 and showing what happens when the test fluid is devoid of antibodies. Specifically, a fluid, generally designated 48, is applied to the surface 30 of layer 12 of the device 40. As the fluid diffuses the matrix of layer 12, the enzyme-linked antigens 36 and the bacterial collagenase 25 are solubilized (FIGURE 6A). After a short degradation period, the collagen fluid flow barrier is digested by the bacterial collagenase 25, allowing the fluid containing the soluble enzyme-linked

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antigens 36 to diffuse into layer 14 (FIGURE 6B), where the enzyme-linked antigens 36 become attached to the bound and immobilized antibodies 38. Accordingly, no enzyme-linked antigens 36 reach the color forming reagent 17 in the second zone 20 (layer 16) and no color change is observed (FIGURE 6C), indicating that the sample is devoid of antibodies.

#### Description of the Practice of the Invention

The present invention relates to an improved method and device for determining the presence of antigens in sample fluids. Specifically, the present invention is directed to the addition of an enzymatically regulated fluid flow barrier between the first reactive zone and the trapping layer of the immunoassay device of the type disclosed in Liotta, U.S. Patent No. 4,446,232. It has been discovered that the addition of the enzymatically regulated fluid flow barrier enhances the incubation period for the antigen-antibody reaction occurring in the first reactive zone between the enzyme-linked antibodies presented therein and the antigens found in the sample fluid. As a result of the enhanced incubation period, a greater number of antigen-antibody(enzyme-linked) complexes are formed which flow, upon degradation of the collagen fluid flow barrier by the bacterial collagenase present in the first reactive zone, through the trapping layer into the substrate layer to produce a color forming reaction. Hence, since more antigen-antibody(enzyme-linked) complexes are formed, because of the increased incubation period, an enhanced color reaction is produced in the substrate zone, increasing the sensitivity of the device.

The device of the present invention includes (see FIGURE 1) a first reactive zone having a matrix layer

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containing dried or lyophilized bacterial collagenase and enzyme-linked antibodies which are capable of immunologically reacting with the antigens being tested for. Attached to the first reactive zone is a protease type enzyme regulated fluid flow barrier which is composed of a sufficient amount of collagen to allow fluid flow only when degraded by the bacterial collagenase of the first reactive zone. A second reactive zone is attached to the fluid flow barrier having a matrix layer containing bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone (i.e. the trapping layer), and a matrix layer containing material capable of reacting with the enzyme linked to the enzyme-linked antibodies of the first reactive zone to produce a color forming reaction to indicate the presence of antigens in the sample fluid (i.e. the substrate layer).

The operation of the device is as follows (see FIGURES 2A, 2B, and 2C). The fluid sample, containing the antigen being tested for, is placed in contact with the first reactive zone of the device. As the sample fluid diffuses through the first reactive zone, the enzyme-linked antibodies and the bacterial collagenase presented therein become solubilized. The free antigens of the sample fluid then contact and bind with most of the soluble enzyme-linked antibodies to form antigen-antibody(enzyme-linked) complexes with saturated recognition sites. The collagen fluid flow barrier inhibits or prevents the fluid containing the free antigens being tested for, the soluble enzyme-linked antibodies and the antigen-antibody(enzyme-linked) complexes with saturated recognition sites from diffusing out of the first reactive zone and into the second reactive zone. As a result of the increased incubation period, due to the collagen fluid flow barrier preventing the diffusion of the fluid, etc., additional

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antigen-antibody(enzyme-linked) complexes with saturated recognition sites are formed.

While the above antigen-antibody reaction is occurring, the solubilized bacterial collagenase also present in the first reactive zone, begins to degrade the attached collagen fluid flow barrier. After a specified time period, depending upon the collagenase concentration and the collagen composition of the fluid flow barrier, the collagen fluid flow barrier is digested by the collagenase, allowing the fluid containing the remaining soluble enzyme-linked antibodies and antigen-antibody(enzyme-linked) complexes with saturated recognition sites to diffuse into the second reactive zone. In the second reactive zone, the leftover enzyme-linked antibodies become attached to the bound and immobilized antigens present in the trapping layer of the second reactive zone. However, because the antibodies(enzyme-linked) bound to the antigens of the antigen-antibody(enzyme-linked) complexes do not have any available recognition sites, the complexes freely diffuse through the bound and immobilized antigens of the trapping layer into the substrate layer of the second reactive zone where the enzyme linked to the antibodies of the antigen-antibody(enzyme-linked) complexes reacts with the substrate to produce a color forming reaction which indicates the presence of antigens in the sample. The concentration of antigen in the test sample will be reflected in the level of the color reaction over time in the substrate layer. A darker color will indicate a higher concentration of antigen.

If, however, the fluid sample is devoid of free antigens, no color reaction will be produced in the substrate layer of the second reactive zone of the present invention. This is because the enzyme-linked antibodies of the first reactive zone will have free binding sites (i.e.

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no free antigens in the sample fluid to bind with) when the collagen fluid flow barrier is digested by the solubilized collagenase (see FIGURES 3A, 3B, 3C). As the fluid containing the enzyme-linked antibodies diffuses into the trapping layer of the device, the enzyme-linked antibodies with the free binding sites combine with the bound and immobilized antigens of the trapping zone. Thus, no enzyme-linked antibodies reach the color forming substrate layer of the second reactive zone and no color change is observed, indicating the sample fluid is devoid of antigens.

In addition, the technique of the present invention is equally applicable to both the detection of antibodies as well as antigens in sample fluids (see FIGURE 4). The roles of the antigens and the antibodies present in the invention would be simply reversed. For example, when the present invention is adapted for the detection of antibodies, antigens, as opposed to antibodies, linked with enzymes are present with the bacterial collagenase in the first reactive zone and referenced antibodies, as opposed to antigens, are immobilized in the trapping layer of the second reactive zone. The same materials utilized in the first embodiment of the invention, including the collagen fluid flow barrier, are also used in the adapted version of the invention.

The operation of the adapted version for the detection of antibodies in sample fluids is also similar to the first device utilized to detect the presence of antigens in sample fluids (see FIGURES 5A, 5B, and 5C). When a sample fluid containing free antibodies is applied to the adapted device, the enzyme-linked antigens and the bacterial collagenase present in the first reactive zone become solubilized. As the bacterial collagenase digests the collagen fluid flow barrier, antibody-antigen(enzyme-

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linked) complexes with saturated recognition sites are formed between the antibodies present in the sample fluid and the enzyme-linked antigens of the first reactive zone. As a result of the increased incubation period due the presence of the fluid flow barrier, a larger amount of antibody-antigen(enzyme-linked) complexes with saturated recognition sites are formed. After a specified period of time, depending once again upon the concentration of the collagenase and the collagen composition of the fluid flow barrier, the collagen fluid flow barrier is digested by the collagenase, allowing the fluid containing the remaining enzyme-linked antigens and the antibody-antigen(enzyme-linked) complexes with saturated recognition sites to diffuse into the second reactive zone, where the leftover enzyme-linked antigens bind with the immobilized antibodies of the trapping layer. The fluid containing the antibody-antigen(enzyme-linked) complexes with saturated recognition sites freely diffuses through the trapping layer into the substrate layer of the second reactive zone where the enzyme linked to the antigen of the antibody-antigen(enzyme-linked) complexes reacts with the substrate to produce a color forming reaction which indicates the presence of antibodies in the sample fluid.

Moreover, the operation of the adapted device for sample fluids devoid of free antibodies is also similar to that described above. No color forming reaction will occur in the substrate layer because the enzyme-linked antigens of the first reactive zone will diffuse, upon digestion of the collagenase fluid flow barrier, into the trapping layer of the second reactive zone where all of the immobilized antibodies present therein will bind with the enzyme-linked antigens. Since no free antibodies are present in the sample fluid, no antibody-antigen(enzyme-linked) complexes



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will be formed. Hence, no color transformation will occur in the substrate layer of the second zone.

The materials utilized to fabricate the first and second reactive zones of the present invention are similar to those disclosed in Liotta, U.S. Patent No. 4,446,232. The zones may be constructed from interwoven fibers such as cellulose, nitrocellulose, methylcellulose, or diazobenzylloxymethyl (DBM) paper, and/or Pall nylon or polyvinylidene difluoride membranes. Nitrocellulose paper directly binds proteins and has been shown to be useful for immobilizing antigens. DBM paper binds DNA, RNA, and proteins by means of covalent linkages to the diazonium group. Nylon and polyvinylidene difluoride membranes have been found useful for binding proteins including IgG.

Furthermore, zone 1 can be an open reservoir containing lyophilized bacterial collagenase and enzyme linked antibodies. The outflow from the bottom of the reservoir which leads to the trapping zone is blocked by the fluid flow barrier.

In addition, porous gels resistant to enzyme degradation by bacterial collagenases, such as polyacrylamide or agarose can be utilized to fabricate the reactive zones. The antigens can be trapped within the pores of the gel, or they can be cross-linked to the gel via amino groups of the ligand and carboxylic groups of the matrix. Similarly, particles or beads containing the bound ligands trapped within a cellulose or plastic fiber matrix can also be used. A satisfactory example is polyacrylamide beads, 5-10 microns in diameter, with antigens bound to the surface via peptide bonds. The beads are trapped within a cellulose filter matrix of pore size 1-2 microns.

The fluid flow barrier may be constructed out of collagen which is the main protein component of bone, cartilage and connective tissue. The collagen layer

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prevents the fluid present in the first reactive zone from diffusing into the second reactive zone of the device. A suitable collagen matrix is that formed by bovine type I collagen in triple helical (native) or denatured (gelatin) form. The collagen can be used alone or with an additive of sugar polymer, cellulose, or non-collagenous proteins.

The collagen barrier can be formed as a dry sheet or coated onto one side of either the first reactive zone or the trapping layer of the second reactive zone. The collagen barrier can also be impregnated into a porous material.

Although collagen is generally resistant to enzyme degradation, some bacteria (e.g. Clostridium perfringens and Clostridium histolyticum) produce specific collagenases which are capable of recognizing amino acid sequences specific for collagen and degrading collagen into small peptides. The preferred bacterial collagenase utilized in the instant invention is that produced by the microorganism, Clostridium histolyticum, which can be obtained from commercial sources such as Boehringer Mannheim.

The present invention incorporates the collagen fluid flow barrier and the specific bacterial collagenases set forth above into the device in order to act as a built-in delay mechanism for allowing the fluid present in the first reactive zone, to diffuse, upon degradation of the collagen barrier, into the second reactive zone of the device. The amount of time required for the degradation of the collagen fluid flow barrier depends on the concentration of the collagenase present in the first reactive zone, as well as the composition and thickness of the collagen layer. In this regard, the following degradation periods were determined for various

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concentrations of bacterial collagenase and various thicknesses of collagen layers:

5	<u>Collagenase</u> <u>Concentration</u>	Degradation Time 0.1 mm collagen <u>barrier</u>	Degradation Time for 0.25 mm <u>collagen barrier</u>
	0.05% w/v	2 min. 55 sec.	3 min. 30 sec.
	0.10% w/v	1 min. 35 sec.	2 min. 20 sec.
	0.20% w/v	38 sec.	1 min. 40 sec.
	0.50% w/v	16 sec.	52 sec.

10           Hence, the amount of bacterial collagenase present in the first reactive zone and the thickness of the collagen fluid flow barrier utilized for each device, determines or regulates the amount of increased incubation time desired.

15           Suitable substrates or color forming reagents are well known in the art. In this regard, a number of different types of purified enzymes are commonly labeled reagent for immunoassays, such as ELISA. These include horseradish peroxidase, alkaline phosphates, and beta-galactosidase. However, the present invention is not  
20 limited to the use of these enzymes to label the antibody or antigen. Other enzyme substrates combinations well known in the art may also be utilized.

25           In addition, the enzyme used to label the antibodies or antigens can produce a color in the second reactive zone of the test device by acting directly or indirectly with the color forming agent. For example, substrates well known in the art such as diaminobenzidine or p-nitrophenylphosphate react with horseradish peroxidase in  
30 the presence of hydrogen peroxide to form a brown to black color. In one form of the present invention, hydrogen

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peroxide is added exogenously during the use of the device. Alternately, the lyophilized color forming substrate layer can contain bound glucose oxidase and the first reactive zone can contain glucose. Upon use of the device, the glucose in the first reactive zone diffuses upon degradation of the collagen fluid flow barrier into the second reactive zone and generates hydrogen peroxide by reacting with the glucose oxidase.

The following are specific examples which further illustrate the practice of the instant invention.

Example 1: Utilization of a layered immunoassay device containing a fluid flow barrier for the detection of theophylline in biological samples.

A. Preparation of theophylline hapten and antibodies thereto

5,6-diamino-1, 3-dimethyl uracil hydrate was reacted with glutaric anhydride by refluxing for 3 hours under nitrogen with N, N-dimethyl aniline using a Dean-Stark trap and condenser. The mixture was cooled, crystallized and washed with benzene. This precipitate was further purified by dissolving in deionized water and crystallized three times. This material was held at 4 degrees C for four hours and dried in an oven at 100 degrees C. This stable intermediate (8-carboxylpropyl theophylline) was attached to bovine serum albumin (BSA) by carbodiimide mediated coupling using 1-ethyl-3(3-dimethyl amino propyl) carbodiimide (EDC). The resulting product (8-carboxypropyl theophylline BSA) was dialyzed in deionized water and lyophilized.

The theophylline hapten produced by the above reactions is then injected into mammals which are repeated

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immunized. The mammals are then sacrificed and the antiserum is collected and purified by affinity chromatography utilizing controlled pore glass (CPG) the solid matrix. Preactivated horseradish peroxidase is then injected into the affinity column and reacted with the purified antibodies. After an appropriate incubation time, excess enzyme is eluted with phosphate buffer from the affinity column. The theophylline antibodies linked with the enzyme are then eluted from the affinity column and collected for use with the present invention.

B. Preparation of antibody enzyme conjugate zones containing various concentrations of collagenase (The First Reactive Zone)

A Tris buffer solution (pH 7.6) containing 0.05 M Tris, 0.2 M NaCl, 0.01 M  $\text{CaCl}_2$ , 2% w/v ovalbumin and 5 ug/ml of the anti-theophylline antibody peroxidase conjugate produced above, was mixed with various concentrations (i.e. from 0 to 0.5% w/v) of a bacterial collagenase obtained from Clostridium histolyticum (0.28 u/mg, Boehringer Mannheim 103578). Pieces of cellulose filter paper (coarse grade) were then impregnated with the antibody enzyme conjugate solutions containing the various concentrations of collagenase by dipping pieces of the filter paper into the solutions and allowing the pieces to dry for 40 minutes in an oven at 30 degrees C.

C. Preparation of the fluid flow barrier

Bovine type I collagen was dissolved in distilled water at a concentration of 6.25% w/v. The dried collagen was dissolved by the slow addition of the collagen to the water which had been previously heated to 90 degrees C.

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When the collagen was dissolved, the solution was poured into a mold and cooled to 4 degrees C to form a collagen gel 1.0 mm in thickness. The gel was submerged in liquid nitrogen and then inserted into a chamber of a lyophilizer and freeze dried for 5 hours. The resulting dried layer was approximately 0.25 mm in thickness.

D. Preparation of the trapping layer containing the immobilized antigen

The theophylline-BSA hapten produced above was solubilized at a concentration of 10 mg/ml in phosphate buffered saline (PBS) at a pH of 7.2. A protein binding membrane of the derivatized nylon variety was immersed into the protein solution for 2 hours. The membrane was then removed, rinsed, and dried for 20 minutes in an oven at 40 degrees C.

E. Preparation of the color forming signal layer containing the substrate material

Cellulose filter paper (coarse grade) was immersed in a solution containing 200 u/ml of glucose oxidase and 0.1% w/v of triton X-100 in acetate buffer (0.1 M  $\text{NaH}_2\text{PO}_4$ , and 0.05 M citric acid, pH 5.5). The filter paper was dried for 30 minutes in an oven at 40 degrees C and then immersed into a second solution containing 2 mg/ml tetramethyl benzidine and 15 mg/ml of B-D glucose in methanol and dried for one minute in an oven at 100 degrees C.

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F. Preparation of the layered immunoassay device  
containing a fluid flow barrier

The dried layers were cut into squares or circles of the following dimensions:

- |    |  |                  |
|----|--|------------------|
| 5  | Antibody-enzyme conjugate zones<br>containing collagenase<br>(The first reactive zone) | 8 mm dia. circle |
|    | Collagen Fluid Flow Barrier  | 8 mm dia. circle |
| 10 | Trapping Layer containing<br>immobilized antigen<br>(The second reactive zone)         | 1 cm square      |
|    | Color forming Substrate Layer<br>(The second reactive zone)                            | 8 mm square      |

- 15 The device was then constructed by attaching the side of each layer in reverse order to a piece of polycarbonate plastic having on one side a strip of double-sided clear micro-industrial tape. For example, the substrate layer was first attached on one side via the double-sided tape to the plastic strip. The trapping layer
- 20 containing the immobilized antigens was then laid on top of the substrate layer and attached on its side to the plastic strip. Next, the collagen fluid flow barrier was laid on top of the trapping layer and attached on its side to the plastic strip. Lastly, the first reactive zone containing
- 25 the collagenase and the antibody-enzyme conjugates was laid on top of the collagen layer and attached on one side to the plastic strip.

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The plastic strip containing the attached layers was then covered with micro-industrial tape to prevent excess fluid flow. A small hole for receiving the sample fluid was inserted into the tape covering the first reactive zone.

G. Utilization of the layered immunoassay devices containing the fluid flow barriers for the detection of theophylline in biological samples

For comparison purposes, human serum samples containing theophylline assayed at 0, 0.5, and 15 ug/ml were utilized to determine the effectiveness of the collagen fluid flow barrier in test strips containing various concentrations of collagenase in the antibody-enzyme conjugate zone (i.e. the first reactive zone). To perform the comparison assays, approximately 50 microliters of the human serum samples containing the various amounts of theophylline were applied to the surface of the first reactive zone (i.e. the antibody-enzyme conjugate zone containing the collagenase) of the test strips fabricated as described above. The amount of time required by the collagenase to degrade the collagen fluid flow barrier was determined for each zone. In addition, the color density of the substrate layer for each test strip was also determined after 2 (two) minutes. The results obtained are set forth below.



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TABLE 1

Concentration of	Time Required for the fluid flow barrier to break down at 20°C	Reflectance Density <sup>1</sup> (Blue Color) of the samples containing Theophylline assayed at:		
		0 ug/ml	0.5ug/ml	15 ug/ml
Collagenase				
0	Indefinite	0 <sup>2</sup>	0 <sup>2</sup>	0 <sup>2</sup>
0.05%	4 min. 19 sec.	0	0.31	0.64
0.10%	1 min. 2 sec.	0	0.22	0.53
0.50%	15 sec.	0	0.12	0.40

<sup>1</sup>in color density units

<sup>2</sup> 0 color reflectance density is defined as a color reaction not distinguishable from background color reaction in the absence of sample antigen in which the maximum amount of free antibodies were trapped in the trapping zone.

5 The above results clearly indicate that the sensitivity of the test strips for the detection of theophylline in test samples increases as a result of the extended incubation periods for the antigen-antibody reaction to occur in the first reactive zone. For example, the sensitivity of the test strips for the detection of theophylline present at 0.5 ug/ml in the sample fluids, increased from 0.12 color density units at an incubation period of 15 seconds to 0.31 color density units at an incubation period of 4 minutes and 19 seconds. This is a significant sensitivity difference since the color device

10

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units are related to the log of the antibody concentration.

The increase in the incubation periods is due to the addition of the collagen layer and the collagenase enzyme to the first reactive zone of the test strip. The collagen layer acts as a fluid flow barrier between the first reactive zone (containing the bacterial collagenase and the enzyme-linked antibodies which are capable of immunologically reacting with the antigens of the second reactive zone) and the second reactive zone (containing immobilized antigens and the color forming substrate) of the test strip. As a result of this barrier, the incubation period for the antigen-antibody reactions to occur in the first reactive zone between the antigens existing in the test sample and the enzyme-linked antibodies present in the test strip is extended, depending upon the amount of collagenase enzyme present in the first layer and the composition of the collagen fluid flow barrier. Because an increased number of enzyme-linked antibodies bind with the antigens present in the test sample, a greater number of enzyme-linked antibody-antigen complexes form and flow, upon breakdown of the collagen layer by the enzyme collagenase, through the first reactive zone into the second reactive zone to produce a color forming reaction which indicates the presence of the antigens being tested for. Hence, as a result of the addition of the collagen fluid flow barrier and the collagenase enzyme to the first reactive zone, the specificity of the test strip is greatly increased.

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Example 2      Utilization of a layered immunoassay  
device containing a fluid flow  
barrier for the detection of human  
chorionic gonadotropin (hCG) in  
biological samples (Pregnancy Test)

5

A.    Preparation of purified Beta hCG antigen and  
antibodies thereto

10      The hCG antigen standard was obtained from  
Monoclonal Antibodies, Inc., lot 10841, 50 mIU/ml hCG  
positive calibrator. The conjugated anti hCG antibodies  
were obtained from Genesis Labs, Inc. (Minneapolis, MN)  
The method of conjugation was as set forth in Example 1.

15

B.    Preparation of the enzyme-linked antibody zone  
containing the bacterial collagenase enzyme  
(The First Reactive Zone)

20

This zone was fabricated as set forth in Example 1  
except the enzyme linked antibodies utilized were the  
horseradish peroxidase conjugated anti-hCG monoclonal  
antibodies indicated above, and the collagenase enzyme  
concentration was approximately 0.05% w/v.

C.    Preparation of the fluid flow barrier  
As set forth in Example 1.

25

D.    Preparation of the trapping layer containing  
the immobilized antigen (The Second Reactive  
Zone)

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The trapping zone was fabricated from the nylon based membrane utilized in Example 1 except that the antigen used was approximately 1000 mIU of Beta hCG.

5        E.    Preparation of the color forming signal layer containing the substrate material (The Second Reactive Zone)

As set forth in Example 1.

10       F.    Fabrication of the layered immunoassay device containing the fluid flow barrier and the utilization of the device for the detection of hCG antigen in biological samples

15       The components of the layered immunoassay device were assembled according to Example 1, except that the collagenase enzyme concentration of the first reactive zone was consistently about 0.05% w/v in each test strip. For comparison purposes, 50 microliters of a test sample containing hCG antigens (50 mIU) and 50 microliters of a test sample devoid of hCG antigen were applied to both a layer immunoassay device containing a fluid flow barrier and a layer immunoassay device lacking such a barrier. The amount of time required for the fluid to enter the substrate layer of each test strips, as well as the color density of the substrate layer, were determined and set forth below.

20

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	<u>Barrier Present</u>	<u>Barrier Absent</u>
5	Time required for the fluid to reach the substrate layer	
	3.25 min.	< 15 sec.
	Color density of the substrate zone	
	A. hCG absent	0.12, 0.11(neg.) 0.14, 0.10(neg.)
10	B. hCG present	0.29, 0.34 0.18, 0.17
	(Strong blue)	(Pale Blue)

The above results indicate that the fluid flow barrier increases the incubation period for the antigen-antibody reaction to occur in the first reactive zone of the test strips. As a result of the increased incubation period, the sensitivity of the assays to determine the presence of hCG antigen in test samples is also increased. For example, when only 50 mIU was present in the test sample, a strong positive blue color was exhibited in the assay containing the fluid flow barrier. However, when the sample containing the hCG was tested with the assay lacking the fluid flow barrier, a weak blue color was observed. Hence, the addition of the collagen fluid flow barrier and the corresponding collagenase enzyme to the layered immunoassay devices, increased the incubation time from 15 seconds to 3.25 minutes, resulting in a significant increase in sensitivity.

While there has been described what are at present considered to be the preferred embodiments of this invention, it will be obvious to those skilled in the art that various changes and modifications may be made therein without departing from the invention, and it is, therefore, aimed in the appended claims to cover all such changes and modifications as fall within the true spirit and scope of the invention.

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What is claimed is:

1. A device for detecting the presence of antigens in a sample fluid which comprises:

5 a) a first reactive zone including a matrix layer containing bacterial collagenase and enzyme-linked antibodies, wherein said antibodies are capable of immunologically reacting with the antigens being tested for;

10 b) a fluid flow barrier operably connected to the first reactive zone for inhibiting the flow of fluid through said first reactive zone, wherein said barrier is composed of a sufficient amount of collagen to allow fluid flow when degraded by the bacterial collagenase of the first reactive zone;

15 c) a second reactive zone operably connected to the fluid flow barrier including (i) a matrix layer containing bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone; and (ii) a matrix layer containing  
20 material capable of reacting with the enzyme linked to the enzyme-linked antibodies to produce a color forming reaction.

25 2. The device of claim 1 wherein said first reactive zone and said fluid flow barrier are in a juxtapositional relationship.

3. The device of claim 1 wherein said fluid flow barrier and said second reactive zone are in a juxtapositional relationship.

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4. The device of claim 1 wherein said first reactive zone is fabricated from nitrocellulose.

5. The device of claim 1 wherein said second reactive zone is fabricated from nitrocellulose.

5 6. The device of claim 1 wherein said second reactive zone is fabricated from a nylon membrane.

7. The device of claim 1 wherein said bacterial collagenase is present in the first reactive zone is obtained from Clostridium histolyticum.

10

8. The device of claim 7 wherein said collagenase is present at a concentration of 0.05% w/v.

9. The device of claim 1 wherein said collagen is bovine type I collagen.

15

10. The device of claim 1 wherein said collagen fluid flow barrier is 0.5 mm in thickness.

11. The device of claim 1 wherein said enzyme-linked antibodies are horseradish peroxidase conjugated anti-hCG antibodies.

20

12. The device of claim 1 wherein said immobilized antigens are hCG antigens.

13. The device of claim 1 wherein said enzyme-linked antibodies are horseradish peroxidase conjugated anti-theophylline antibodies.

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14. The device of claim 1 wherein said immobilized antigens are theophylline antigens.

15. A method for detecting the presence of antigens in a sample fluid which comprises the steps of:

5 bringing a fluid which is to be tested for the presence of antigens into contact with a device including

a) a first reactive zone having a matrix layer containing bacterial collagenase and enzyme-linked antibodies, wherein said antibodies are capable of immunologically reacting with the antigens being tested for;

10 b) a fluid flow barrier operably connected to the first reactive zone for inhibiting the flow of fluid through said first reactive zone, wherein said barrier is composed of a sufficient amount of collagen to allow fluid flow when degraded by the bacterial collagenase of the first reactive zone;

15 c) a second reactive zone operably connected to the fluid flow barrier having (i) a matrix layer containing bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone; and (ii) a matrix layer containing material capable of reacting with the enzyme linked to enzyme-linked antibodies to produce a color forming reaction;

20 allowing said fluid to permeate the device; and, observing the presence or absence of any color change in the second reactive zone to thereby determine the presence of antigens being tested for in the sample fluid.

25 16. The method of claim 15 wherein said first reactive zone and said fluid flow barrier of the device are in a juxtapositional relationship.



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17. The method of claim 15 wherein said fluid flow barrier and said second reactive zone of the device are in a juxtapositional relationship.

5 18. The method of claim 15 wherein said first reactive zone of the device is fabricated from nitrocellulose.

19. The method of claim 15 wherein said second reactive zone of the device is fabricated from nitrocellulose.

10 20. The method of claim 15 wherein the antigen being tested for is hCG and wherein the antigen immobilized in said device is human chorionic gonadotrophic.

15 21. The method of claim 15 wherein the antigen being tested for is theophylline and wherein the antigen immobilized in said device is theophylline.

22. A device for detecting the presence of antibodies in a sample fluid comprises:

20 a) a first reactive zone having a matrix layer containing bacterial collagenase and enzyme-linked antigens, wherein said antigens are capable of immunologically reacting with the antibodies being tested for;

25 b) a fluid flow barrier operably connected to the first reactive zone wherein said barrier is composed of a sufficient amount of collagen to allow fluid flow when degraded by the bacterial collagenase of the first reactive zone;

c) a second reactive zone operably connected to the fluid flow barrier having (i) a matrix layer containing

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bound and immobilized antibodies which are of the type recognized by the enzyme-linked antigens of the first reactive zone; and (ii) a matrix layer containing material capable of reacting with the enzyme linked to the enzyme-linked antigens to produce a color forming reaction.

23. The device of claim 22 wherein said first reactive zone and said fluid flow barrier are in a juxtapositional relationship.

24. The device of claim 22 wherein said fluid flow barrier and said second reactive zone are in a juxtapositional relationship.

25. The device of claim 22 wherein said first reactive zone is fabricated from nitrocellulose.

26. The device of claim 22 wherein said second reactive zone is fabricated from nitrocellulose.

27. The device of claim 22 wherein said second reactive zone is fabricated from a nylon membrane.

28. The device of claim 22 wherein said bacterial collagenase is present in the first reactive zone and obtained from Clostridium histolyticum.

29. The device of claim 28 wherein said collagenase is present at a concentration of 0.05% w/v.

30. The device of claim 22 wherein said collagen is bovine type I collagen.

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31. The device of claim 22 wherein said collagen fluid flow barrier is 0.5 mm in thickness.

32. A method for detecting the presence of antibodies in a sample fluid which comprises the steps of:

5 bringing a fluid which is to be tested for the presence of antibodies into contact with a device having

10 a) a first reactive zone having a matrix layer containing bacterial collagenase and enzyme-linked antibodies, wherein said antigens are capable of immunologically reacting with the antibodies being tested for;

15 b) a fluid flow barrier operably connected to the first reactive zone, wherein said barrier is composed of a sufficient amount of collagen to allow fluid flow when degraded by the bacterial collagenase of the first reactive zone;

20 c) a second reactive zone operably connected to the fluid flow barrier having (i) a matrix layer containing bound and immobilized antibodies which are of the type recognized by the enzyme-linked antigens of the first reactive zone; and (ii) a matrix layer containing material capable of reacting with the enzyme linked to the enzyme-linked antigens to produce a color forming reaction;

allowing said fluid to permeate the device;

25 observing the presence or absence of any color change in the second reactive zone to thereby determine the presence of the antibodies being tested for in the sample fluid.

33. The method of claim 32 wherein said first reactive zone and said fluid flow barrier of the device are in a juxtapositional relationship.

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34. The method of claim 32 wherein said fluid flow barrier and said second reactive zone are in a juxtapositional relationship.

5 35. The method of claim 32 wherein said first reactive zone of the device is fabricated from nitrocellulose.

36. The method of claim 33 wherein said second reactive zone of the device is fabricated from nitrocellulose.

10 37. A device for detecting the presence of antigens in a sample fluid which comprises:

15 a) a first reactive zone containing bacterial collagenase and enzyme-linked antibodies, wherein said antibodies are capable of immunologically reacting with the antigens being tested for;

20 b) a fluid flow barrier operably connected to the first reactive zone for inhibiting the flow of fluid through said first reactive zone, wherein said barrier is composed of a sufficient amount of collagen to allow fluid flow when degraded by the bacterial collagenase of the first reactive zone;

25 c) a second reactive zone operably connected to the fluid flow barrier including (i) a matrix layer containing bound and immobilized antigens which are of the type recognized by the enzyme-linked antibodies of the first reactive zone; and (ii) a matrix layer containing material capable of reacting with the enzyme linked to the enzyme-linked antibodies to produce a color forming reaction.

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38. The device of claim 37 wherein said first reactive zone and said fluid flow barrier are in a juxtapositional relationship.

5 39. The device of claim 37 wherein said fluid flow barrier and said second reactive zone are in a juxtapositional relationship.

1/2

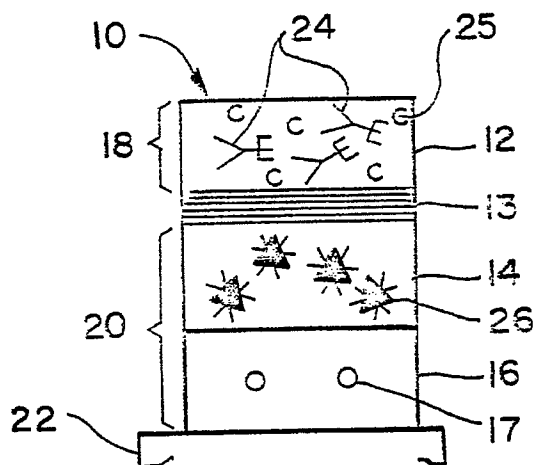


FIG. 1

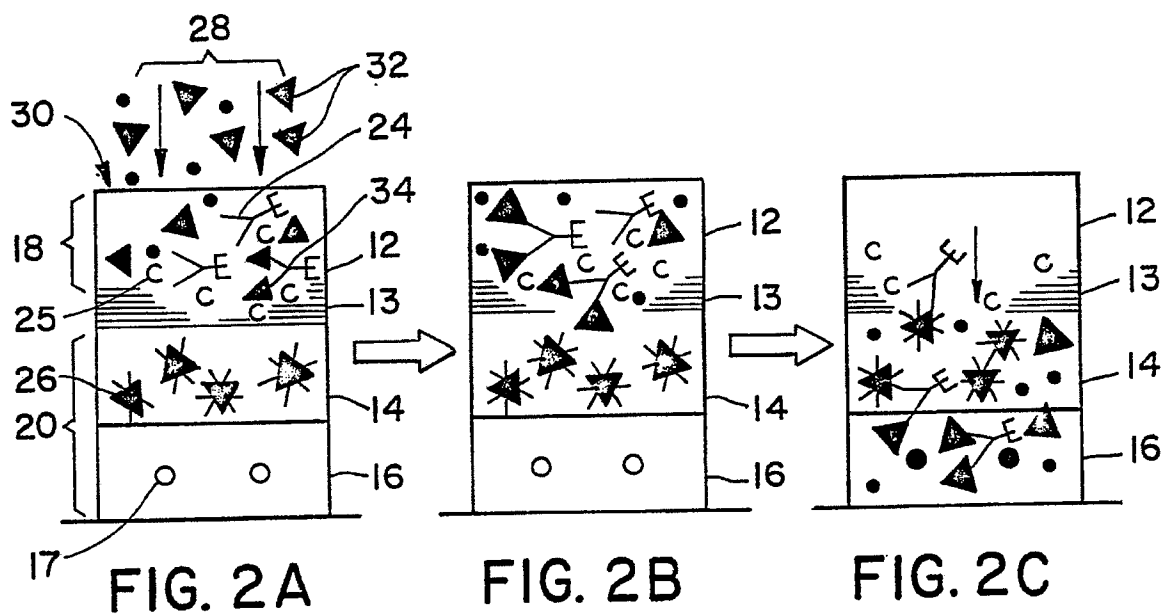


FIG. 2A

FIG. 2B

FIG. 2C

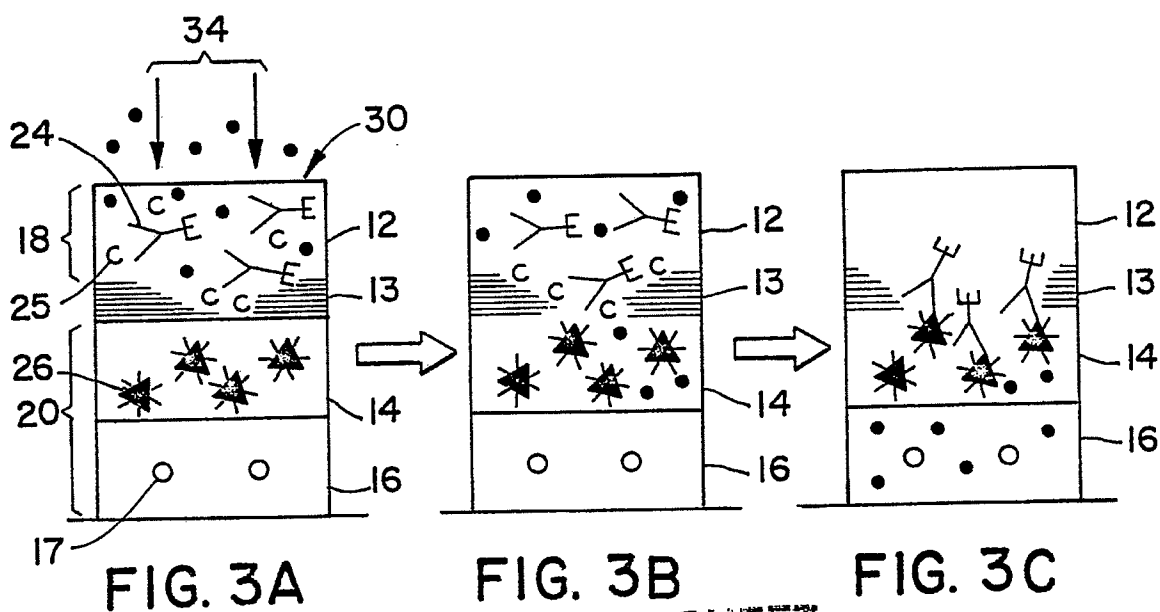


FIG. 3A

FIG. 3B

FIG. 3C

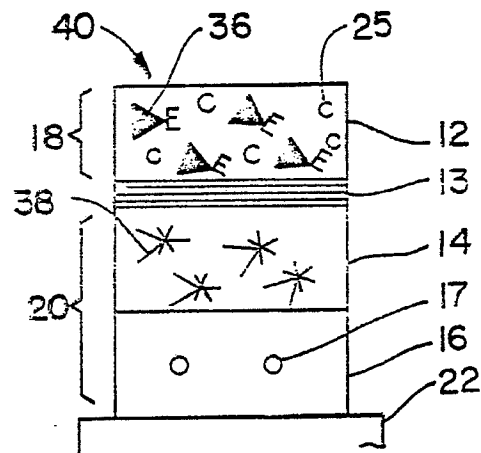


FIG. 4

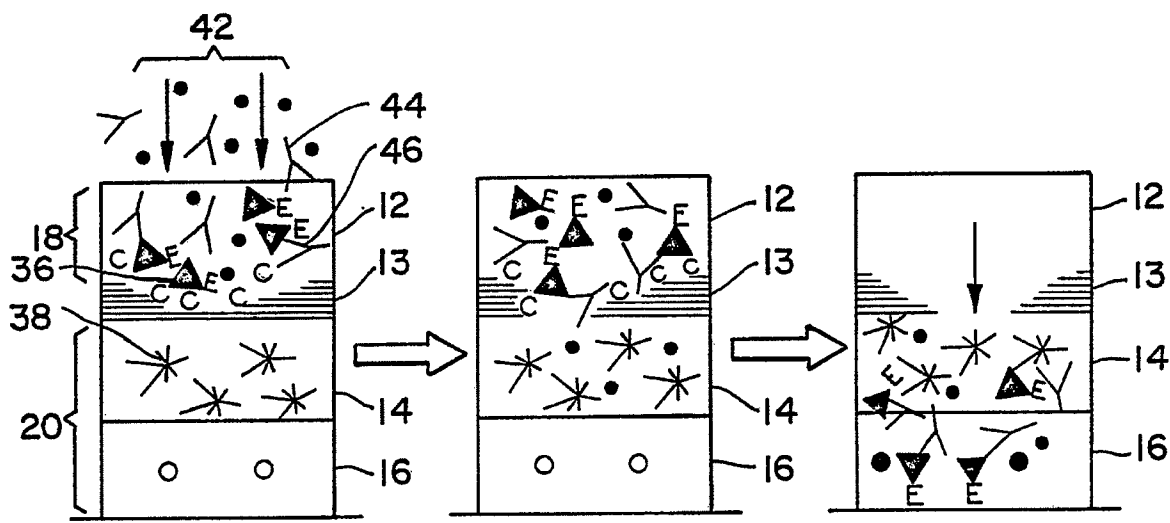


FIG. 5A

FIG. 5B

FIG. 5C

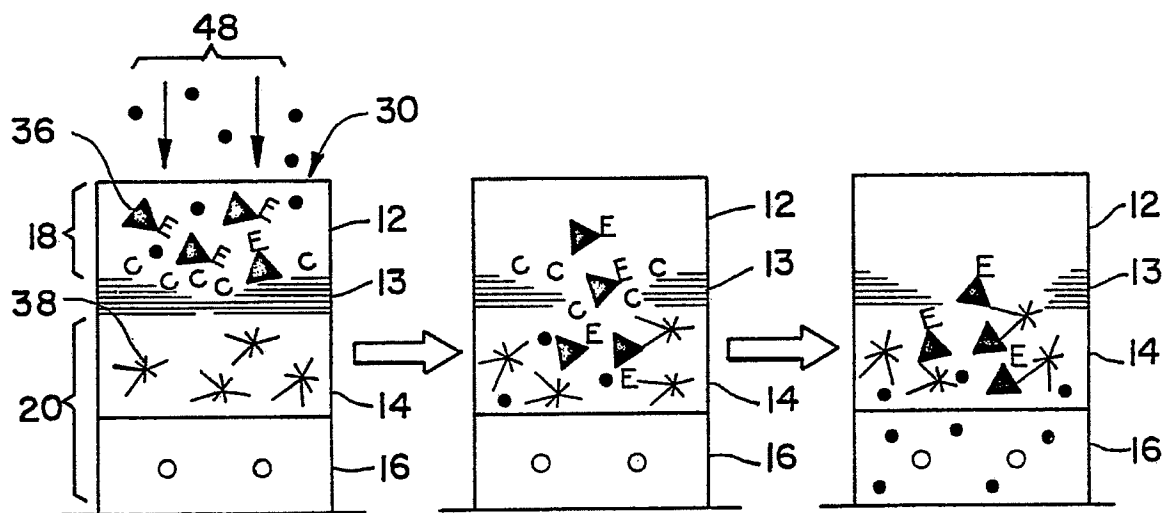


FIG. 6A

FIG. 6B

FIG. 6C

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US88/011159**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC(4): <b>G01N 33/53, 33/543, 33/558, 33/566, 31/22</b> U.S. Cl.: <b>435/4, 7, 805; 436/501, 514, 518, 170, 807; 422/55, 60</b>														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="vertical-align: top; border-right: 1px solid black; padding: 5px;"><b>U.S.</b></td> <td style="padding: 5px;"><b>435/4, 7, 174, 177, 288, 805, 810; 436/501, 514, 518, 170, 807, 810; 422/55, 56, 58, 60, 61</b></td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div> <p style="padding: 10px 0 0 40px;">Computer Search: <b>APS</b></p>			Classification System	Classification Symbols	<b>U.S.</b>	<b>435/4, 7, 174, 177, 288, 805, 810; 436/501, 514, 518, 170, 807, 810; 422/55, 56, 58, 60, 61</b>								
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; text-align: left; padding: 5px;">Category <sup>*</sup></th> <th style="width: 60%; text-align: left; padding: 5px;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 30%; text-align: left; padding: 5px;">Relevant to Claim No. <sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>A</b></td> <td style="padding: 5px;"><b>U.S., A, 4,446,232 (LIOTTA) 01 May 1984, see Abstract and column 5, lines 13-28.</b></td> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>1-39</b></td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>A</b></td> <td style="padding: 5px;"><b>U.S., A, 4,522,786 (EBERSOLE) 11 June 1985, see Abstract, column 4, line 57 - column 5, line 15 and column 6, line 24 - column 7, line 24.</b></td> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>1-39</b></td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>A</b></td> <td style="padding: 5px;"><b>U.S., A, 4,587,102 (NAGATOMO) 06 May 1986, see column 12, lines 49-65.</b></td> <td style="text-align: center; vertical-align: top; padding: 5px;"><b>1-39</b></td> </tr> </tbody> </table>			Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	<b>A</b>	<b>U.S., A, 4,446,232 (LIOTTA) 01 May 1984, see Abstract and column 5, lines 13-28.</b>	<b>1-39</b>	<b>A</b>	<b>U.S., A, 4,522,786 (EBERSOLE) 11 June 1985, see Abstract, column 4, line 57 - column 5, line 15 and column 6, line 24 - column 7, line 24.</b>	<b>1-39</b>	<b>A</b>	<b>U.S., A, 4,587,102 (NAGATOMO) 06 May 1986, see column 12, lines 49-65.</b>	<b>1-39</b>
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<b>A</b>	<b>U.S., A, 4,587,102 (NAGATOMO) 06 May 1986, see column 12, lines 49-65.</b>	<b>1-39</b>												
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the International filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-right: 1px solid black; padding: 5px;">           Date of the Actual Completion of the International Search   <div style="text-align: center; font-size: 1.2em;">13 March 1989</div> </td> <td style="width: 50%; padding: 5px;">           Date of Mailing of this International Search Report   <div style="text-align: center; font-size: 1.5em;">27 APR 1989</div> </td> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">           International Searching Authority   <div style="text-align: center;">ISA/US</div> </td> <td style="padding: 5px;">           Signature of Authorized Officer  <div style="text-align: center;">              Janelle Graeter           </div> </td> </tr> </table>			Date of the Actual Completion of the International Search  <div style="text-align: center; font-size: 1.2em;">13 March 1989</div>	Date of Mailing of this International Search Report  <div style="text-align: center; font-size: 1.5em;">27 APR 1989</div>	International Searching Authority  <div style="text-align: center;">ISA/US</div>	Signature of Authorized Officer <div style="text-align: center;">              Janelle Graeter           </div>								
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